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METHOD

High-performance liquid chromatography purification of homogenous-length RNA produced by *trans* cleavage with a hammerhead ribozyme

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ABSTRACT

An improved method is presented for the preparation of milligram quantities of homogenous-length RNAs suitable for nuclear magnetic resonance or X-ray crystallographic structural studies. Heterogeneous-length RNA transcripts are processed with a hammerhead ribozyme to yield homogenous-length products that are then readily purified by anion exchange high-performance liquid chromatography. This procedure eliminates the need for denaturing polyacrylamide gel electrophoresis, which is the most laborious step in the standard procedure for large-scale production of RNA by in vitro transcription. The hammerhead processing of the heterogeneous-length RNA transcripts also substantially improves the overall yield and purity of the desired RNA product.

Keywords: hammerhead ribozyme; HPLC; in vitro transcription; RNA synthesis

INTRODUCTION

In vitro transcription with T7 RNA polymerase using DNA templates represents an efficient method for large-scale synthesis of RNA (Milligan et al., 1987; Batey et al., 1992; Nikonowicz et al., 1992). However, RNAs synthesized by in vitro transcription are usually heterogeneous in length because of the addition of one or more noncoded nucleotides at the 3′ end of the RNA (Milligan et al., 1987). The proportion of these longer transcripts (collectively referred to here as N+1 RNAs) is template dependent, but is often equal to or greater than the amount of correct-length RNA. For most nuclear magnetic resonance (NMR) or X-ray structural studies, RNA transcripts must be purified to single-nucleotide resolution. For short RNAs ($\leq$20 nt) anion-exchange high-performance liquid chromatography (HPLC) can be used to produce homogenous-length RNA (Wincott et al., 1995; Anderson et al., 1996), and for medium-length RNAs ($<$50 nt) preparative denaturing polyacrylamide gel electrophoresis (PAGE) is typically used to separate the desired RNA from the N+1 RNA products (Nikonowicz et al., 1992). Neither of these procedures can provide single-nucleotide resolution at lengths $\geq$50 nt and therefore both *trans*- and *cis*-cleaving ribozymes have been used to eliminate 3′ terminal length heterogeneity in longer RNAs produced by T7 transcription (Dzianott & Bujarski, 1988, 1989; Grosshans & Cech, 1991; Price et al., 1995; Ferré D'Amaré & Doudna, 1996).

Here we present an efficient method for synthesizing milligram quantities of homogenous-length medium-sized RNAs utilizing *trans* cleavage with a hammerhead ribozyme (Hutchins et al., 1986; Uhlenbeck, 1987; Stage-Zimmermann & Uhlenbeck, 1998) coupled with HPLC purification. The target RNA is initially transcribed with a 3′-terminal extension of 5–10 nt, which is subsequently removed by cleavage with a hammerhead ribozyme added in *trans* (see Fig. 1A). The 3′-terminal extension effectively displaces the N+1 RNA heterogeneity away from the 3′-terminus of the desired RNA; thus the heterogeneous-length transcription products are all cleaved at a specific site by a hammerhead ribozyme. The hammerhead RNA, the substrate RNA(s),

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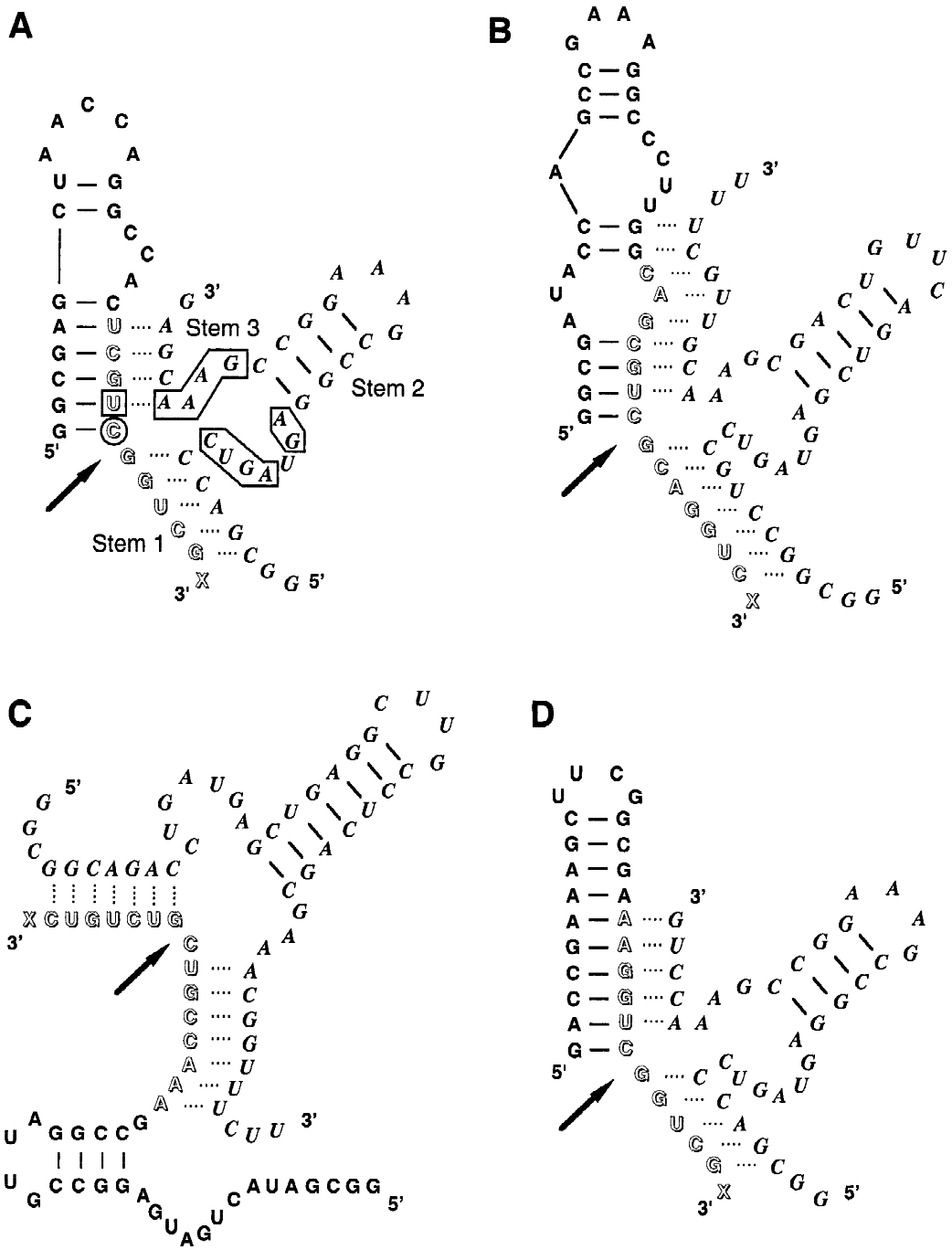

FIGURE 1. Various RNA-substrate *trans*-cleaving hammerhead systems studied in this work. For each substrate RNA, the nucleotides designed to base pair with the processing hammerhead are in outline font, and intermolecular base pairs are designated by solid lines, intramolecular (hammerhead-substrate) base pairs are designated by dashed lines, and the site of cleavage is indicated by the arrow. The *trans*-cleaving hammerhead for each RNA is shown in italic and the N+1 nucleotides at the 3′ end of the RNA substrates are designated by X. In **A**, the 11 conserved nucleotides required for hammerhead activity are boxed, the nucleotide at the cleavage site (which can be any base but G) is circled and stems 1–3 in the hammerhead ribozyme–substrate complex are denoted. **A**: a bFGF-binding RNA; **B**: a theophylline-binding RNA; **C**: a faster-cleaving hammerhead ribozyme variant; and **D**: an RNA that contains specific metal binding sites.

and the product RNA are all designed to differ in length by at least 5 nt so that purification of the RNA product can be accomplished by anion-exchange HPLC. An important advantage of eliminating the 3′ heterogeneity generated by in vitro transcription is that the N+1 RNA transcripts are efficiently converted into correct-length RNA product, leading to much higher overall yields of the desired RNA. Thus *trans* cleavage with a hammerhead ribozyme coupled with HPLC purification leads to higher yields and higher purity of the RNA

while also eliminating the most laborious step in large-scale RNA preparation, the purification of RNA transcripts by denaturing PAGE. The procedure presented here will be compared with other methods for eliminating 3′ heterogeneity in RNAs that are generated by in vitro transcription (Dzianott & Bujarski, 1988; Grosshans & Cech, 1991; Altschuler et al., 1992; Price et al., 1995; Ferré D'Amaré & Doudna, 1996; Lapham & Crothers, 1996; Doudna, 1997).

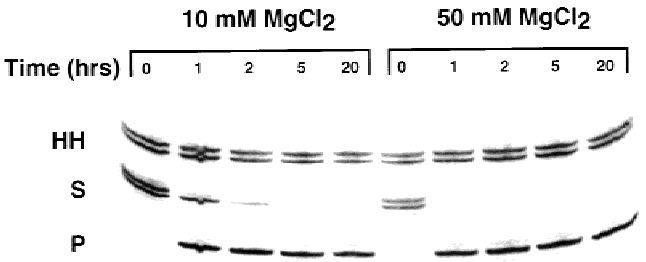

FIGURE 2. 20% PAGE analysis of analytical scale hammerhead cleavage reactions for the bFGF-binding RNA system. Conditions were 50 μM substrate, 50 μM hammerhead, 40 mM Tris, 100 mM NaCl, 0.5 mM EDTA, pH 7.4, 37 °C. Reactions were initiated by addition of either 10 mM or 50 mM $MgCl_2$ and analyzed at the time points shown. Note that both reactions convert the heterogeneous-length substrate RNA into the homogenous-length product form, but that the reaction reaches completion significantly faster at 50 mM $MgCl_2$.

RESULTS AND DISCUSSION

Sequence design requirements for *trans* cleavage with the hammerhead ribozyme

The *trans*-cleaving ribozymes employed here are derived from two well-characterized hammerhead systems, known as HH8 and HH16, (Fedor & Uhlenbeck, 1992; Hertel et al., 1994; Stage-Zimmermann & Uhlenbeck, 1998). The 11 conserved nucleotides required for activity are highlighted in Figure 1, showing that most of the sequence requirements are on the ribozyme. This hammerhead ribozyme can be designed to efficiently cleave RNA substrates at any 5′-UX-3′ (X ≠ G) sequence. Sequence heterogeneity at the 3′-terminus of the substrate RNA will have no effect on the site of hammerhead cleavage; thus the same hammerhead ribozyme will cleave both N (correct length) and N+1 (extra nucleotide(s)) forms of an RNA substrate into identical products. Stems 1 and 3 of the hammerhead ribozyme recognize the substrate RNA and can be changed in sequence and/or length without affecting the site of cleavage. In addition, stem 2 and its accompanying hairpin loop can be altered in sequence and length to tailor the size and/or thermal stability of a hammerhead for a particular system.

Trans cleavage with a hammerhead ribozyme has been used here to produce homogeneous-length RNA in a variety of systems including: an RNA aptamer that binds with high affinity and specificity to basic fibroblast growth factor (bFGF) (Jellinek et al., 1993; Fig. 1A); a theophylline-binding RNA aptamer (Jenison et al., 1994; Fig. 1B); a faster cleaving hammerhead ribozyme variant (Clouet-d'Orval & Uhlenbeck, 1997; Fig. 1C); and an RNA containing specific metal-binding sites (Baeyens et al., 1996; Fig. 1D). In each of these systems the hammerhead ribozyme, substrate, and product RNAs differ in length by at least 5 nt, which allows efficient separation of the RNAs by either PAGE or anion-exchange HPLC.

Optimization and large scale hammerhead cleavage

Figure 2 illustrates the rate of conversion of heterogeneous-length bFGF binding RNA transcripts to homogeneous-length product for analytical scale reactions at two different Mg^{2+}-ion concentrations. The twin bands of the substrate RNA disappear as the RNA is converted into a single-length product. To achieve complete conversion while minimizing nonspecific RNA degradation, the $MgCl_2$ concentration and time required for complete conversion are optimized for each RNA system. The preparative scale hammerhead reactions were typically performed at 25 mM $MgCl_2$ at 37 °C and proceeded to completion in 4 h. We observed the highest yield of cleaved product by using approximately equal molar ratios of hammerhead to substrate. Because the hammerhead is a catalytic RNA, it is also possible to use a lower ratio of ribozyme to substrate, with each ribozyme cleaving multiple substrates. This requires careful design of the sequence and lengths of helices in stems 1 and 3 of the hammerhead, such that the rate of product release is fast enough to allow efficient turnover (Stage-Zimmermann & Uhlenbeck, 1998). In general we were unable to obtain highly efficient cleavage of substrates when catalytic amounts of the hammerhead were used for the *trans* cleavage reactions. One of the reasons for the poor level of cleavage is that in most of the RNAs studied here the residues 5′ to the cleavage site form stable intramolecular helices (see Fig. 1). Thus if stem 3 in the hammerhead is too short, formation of the intermolecular hammerhead–substrate helix will be unfavorable (Stage-Zimmermann & Uhlenbeck, 1998). However if stem 3 is made much longer than ~5–7 nt, product release will become rate limiting, leading to inefficient turnover of the hammerhead and to lower extents of substrate cleavage. Higher rates of turnover can be achieved by raising the temperature, but this also leads to higher rates of nonspecific cleavage of the RNA. Thus, although it is possible to achieve multiple turnover of the hammerhead, this leads to a lower overall yield of the desired product and/or additional nonspecific cleavage of the RNA product. This is especially undesirable for NMR studies with isotopi-

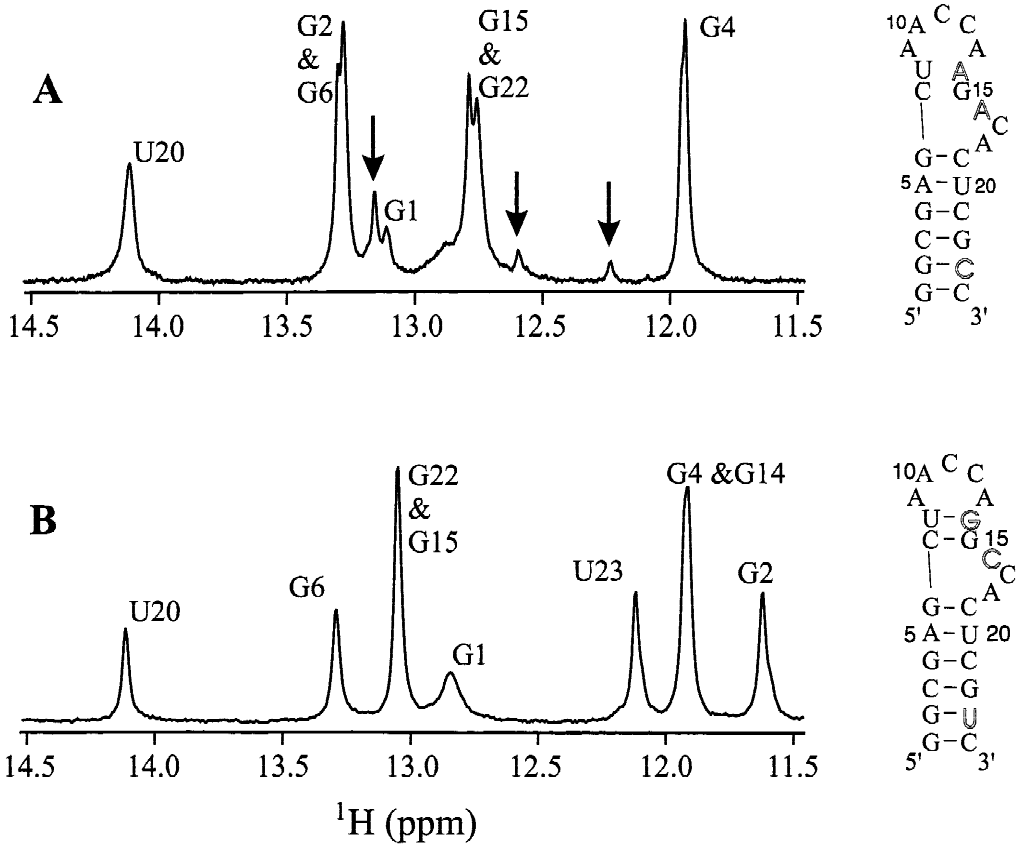

FIGURE 3. ^{1}H NMR spectra of two similar bFGF-binding RNAs, displaying the base-paired imino proton region. Samples were prepared by in vitro transcription where (**A**) the N and N+1 length RNAs were pooled and (**B**) the 3′ length heterogeneity was eliminated by *trans* cleavage with a hammerhead ribozyme. The sequences of these RNAs are given to the right of their spectra and positions that differ between the two RNAs are highlighted. The arrows in **A** denote the extra peaks that arise due to the 3′-terminal heterogeneity in this NMR sample. Although the heterogeneity in **A** could be removed by 20% PAGE under conditions where single-nucleotide resolution is achieved, this results in a significant reduction in the yield of correct length RNA.

cally labeled RNA, where the isotope-labeled precursors are far more expensive than the production of additional hammerhead ribozyme. In our experience, we obtained the highest yield of the desired RNA products using an ~1:1 ratio of ribozyme and substrate, where stems 1 and 3 in the hammerhead-substrate complex were 5–8 nt in length.

Figure 3 displays imino proton spectra for two similar RNAs that are ligands for bFGF (Jellinek et al., 1993), one produced by standard transcription methods and the other by *trans* cleavage with the hammerhead ribozyme. In the RNA produced by standard transcription there are extra peaks due to the presence of N+1 RNA. This heterogeneity is absent in the RNA produced with hammerhead processing. The length heterogeneity observed in Figure 3A can also be removed by preparative denaturing PAGE methods, but this typically leads to a lower overall yield by a factor of 2 to 3 compared to the hammerhead method. In many duplex RNAs, we observe that the 3′ heterogeneity causes chemical shift perturbations of resonances up to three bp in from the terminus. Thus we now use hammerhead processing for large scale production of all RNAs. The yields of various RNA samples prepared by *trans* cleavage with the hammerhead ribozyme are given in Table 1.

HPLC purification and product identification

The purification of RNA by gel electrophoresis is often the rate-limiting step in large-scale production of RNA. Thus we employ HPLC to facilitate purification of RNAs produced by in vitro transcription. It is generally possible to achieve single-nucleotide resolution only for relatively small RNAs (<25 nt) by HPLC. Thus for longer RNAs, denaturing PAGE is the only method for separation of full-length transcripts from N+1 RNA. However the procedure described here eliminates the requirement for single-nucleotide resolution at both the initial transcription and postcleavage purification steps, allowing the use of anion-exchange HPLC methodology to purify homogenous-length RNA. This HPLC method utilizes a medium-capacity anion-exchange column equipped with a column heater, and can achieve

TABLE 1. Yields of NMR samples prepared by the *trans*-cleavage hammerhead method.

RNA[a]	Isotopic label	Transcription reaction size (mL)[b]	Yield of purified RNA (μmol)
bFGF-binding RNA	^{15}N	25	0.53
bFGF-binding RNA	^{13}C, ^{15}N	40	1.1
bFGF-binding RNA	unlabeled	30	0.60
Theophylline-binding RNA	^{15}N	50	1.2
Theophylline-binding RNA	^{13}C, ^{15}N	50	1.3
Faster-cleaving hammerhead RNA	^{15}N	20	0.63
RNA with metal-binding sites	unlabeled	30	0.70

[a] Sequences are given in Figure 1.
[b] Typical transcription conditions are given in Materials and Methods.

suitable resolution of at least 50-nt RNAs at high throughput (~2–5 mg RNA per injection). We observed that the column needs to be heated to 85–90 °C to obtain high-resolution chromatographs. Broad or multiple peaks were observed for some of the pure RNAs when the HPLC was performed at lower temperatures. This is likely due to alternate conformations of RNAs where high temperature denatures the RNA, which then run as a single species on the HPLC. These high temperatures do not appear to significantly affect the properties of the column, as we have used the same HPLC column for several years with no observed degradation in performance.

Figure 4 displays analytical and preparative HPLC traces from the initial purification of the substrate for the bFGF binding RNA. The retention time in preparative injections is reproducibly shorter than the retention time in analytical traces, even though the preparative scale does not appear to overload the HPLC column. For the preparative injections in Figure 4B, the large peak at ~27–30 min was collected in three sequential fractions. These fractions were analyzed by 20% denaturing PAGE and illustrated that this initial HPLC purification only has a resolution of ± ~2 nt (Fig. 4C). This initial purification step readily eliminates the majority of RNA impurities, including smaller aborted transcripts that would comigrate with the desired final RNA product after hammerhead cleavage. Thus a crude HPLC purification of the transcription reaction is all that is required for subsequent processing with the hammerhead ribozyme. Following hammerhead cleavage, the 10-nt length difference between the hammerhead and product RNA allows facile separation by HPLC. An analytical HPLC trace from the final purification (i.e., after hammerhead cleavage) for the bFGF binding RNA is shown in Figure 5A. Figure 5B shows that the large-scale reaction for hammerhead cleavage *in trans* is complete after 4 h, and that the product RNA isolated by anion-exchange HPLC migrates essentially as a single band on a denaturing gel. ^{1}H NMR of this HPLC-purified sample reveals the same high purity as displayed in the spectra of the gel purified sample (data not shown).

Hammerhead cleavage yields a 2′, 3′-cyclic terminal phosphate in the product RNA (Stage-Zimmermann & Uhlenbeck, 1998). Both ^{31}P NMR spectroscopy and MALDI-TOF mass spectroscopy confirm the 2′, 3′-cyclic phosphate on 3′-terminus of the bFGF binding RNA (not shown). The 2′, 3′-cyclic phosphate is relatively stable in standard buffers (e.g., pH 5.5–6.5, 20 mM phosphate, 10–150 mM NaCl, 0.2 mM EDTA) for extended periods of time. However in some cases we observed that the high temperature (88–90 °C) required for HPLC separation of the RNA can lead to partial opening of the 2′, 3′-cyclic phosphate to mixed 2′- and 3′-monophosphates. For production of the faster cleaving hammerhead ribozyme variant substrate (Fig. 1C), HPLC purification following hammerhead cleavage yielded two products with nearly identical HPLC retention times. Although ^{1}H NMR spectra of the two products were very similar, ^{31}P NMR identified the two products as the cyclic and monophosphate termini (data not shown).

If desired, the 3′-terminal cyclic phosphate can be removed by treatment with polynucleotide kinase (PNK), which contains a 3′-phosphatase activity (Weber, 1985). This is illustrated in Figure 5C where 2-h treatment with PNK effectively changes the gel mobility of the RNA, indicating removal of the 2′, 3′-cyclic phosphate. The cyclic phosphate can also be opened under mild acidic conditions (Abrash et al., 1967; Buzayan et al., 1986) to produce mixed 2′- and 3′-monophosphates. The 2′, 3′-terminal phosphate has recently been shown to affect the chemical shifts and exchange rates of imino protons in base pairs near the 3′-terminus of a small hairpin RNA (Alam et al., 1998). In our experience the presence of the 2′, 3′ cyclic or mixed phosphates only affects the chemical shifts of the ribose protons and carbons on the 3′ nucleotide; however, the identity and heterogeneity of the 3′-terminal phosphate should be considered when producing RNAs by cleavage with a hammerhead ribozyme. A combination of ^{31}P NMR, gel mobility on 20% denaturing PAGE, and MALDI mass spectroscopy can be used to determine the chemical identity of the 3′-terminus.

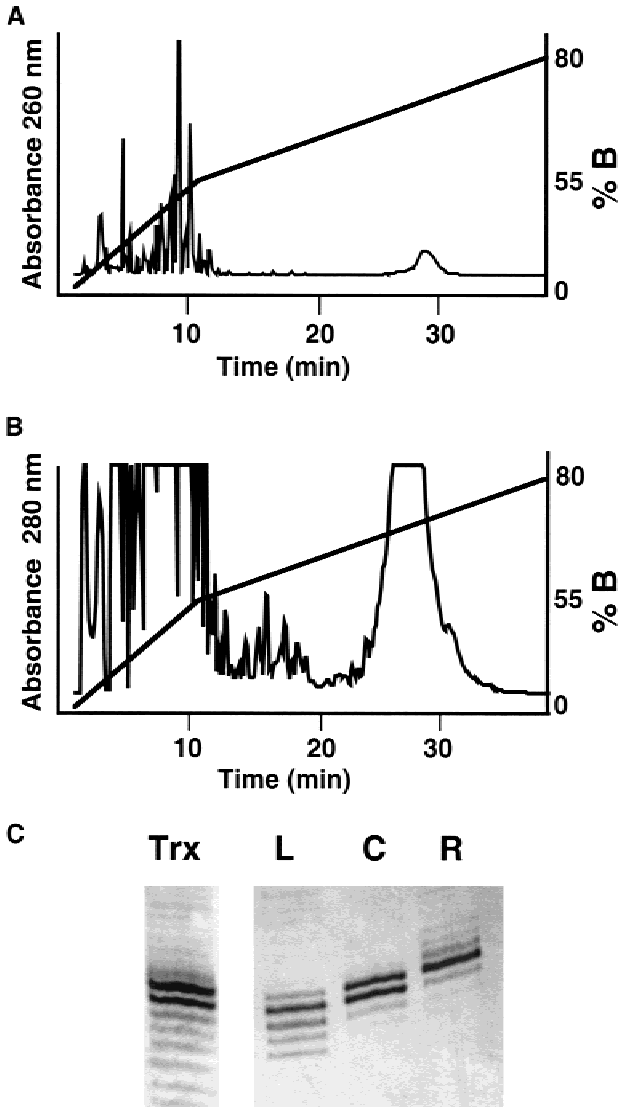

FIGURE 4. Initial purification of bFGF-binding RNA by anion-exchange HPLC. The RNA was transcribed on 30-mL scale, precipitated with ethanol, and resuspended in 20 mM TRIS, 0.5 mM EDTA, pH 8.0 (see text). **A**: Analytical injection containing <1% of the total transcription reaction. **B**: A typical preparative injection (~4 mL of transcription reaction containing 2 mg of RNA). HPLC conditions were: a flow rate of 5 mL/min, column heated to 88 °C, and detection at 260 nm (**A**) or 280 nm (**B**). The gradient (% Buffer B) is given on the right axis. **C**: The upper section of the 20% PAGE on the bFGF-binding RNAs. On the left is the crude transcription reaction (Trx) and on the right are three fractions of the product peak from the preparative HPLC shown in **B**. The full gel of the transcription reaction is shown in Figure 5B. The three fractions correspond to the left, center, and right part of the product peak and are labeled, L, C, and R, respectively.

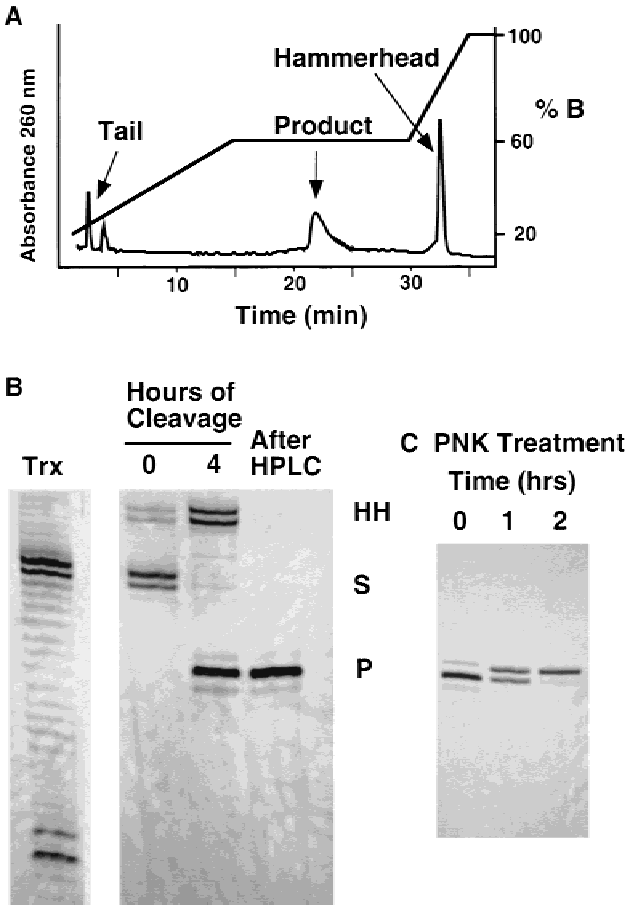

FIGURE 5. Analysis of the large-scale production of bFGF-binding RNA produced by *trans* cleavage with the hammerhead ribozyme. **A**: Analytical HPLC trace displaying the separation of the hammerhead ribozyme, the RNA product, and the cleaved 3′ tail for the bFGF RNA. The gradient (% Buffer B) is given on the right axis. **B**: 20% PAGE analysis of the bFGF-binding RNA. The gel on the left shows the crude transcription reaction (Trx), illustrating that the initial transcription reaction contained significant amounts of aborted transcriptions of the same length as the final RNA product. In the gel on the right, lanes 1 and 2 illustrate the extent of the cleavage reaction and lane 3 illustrates the level of purity after HPLC. HH, S, and P stand for hammerhead, substrate, and product RNAs, respectively. **C**: 20% PAGE analysis of the time course of the reaction with PNK. The change in migration of the RNA following treatment with PNK reflects removal of the 2′, 3′-cyclic phosphate (see text).

Comparison with other methods for generating homogenous-length RNAs produced by in vitro transcription

Various *cis* and *trans* ribozyme systems have been described for eliminating heterogeneity at the 3′ and 5′-termini of in vitro-transcribed RNAs (Dzianott & Bujarski, 1988; Grosshans & Cech, 1991; Altschuler et al., 1992; Price et al., 1995; Ferré D'Amaré & Doudna, 1996). For cleavage *in cis*, the RNA is usually transcribed from a plasmid or PCR DNA template that contains a *cis*-cleaving ribozyme at the 3′-terminus of the RNA (and also at the 5′-terminus if desired). This procedure is better suited for longer RNAs, because both the transcription and *cis* cleavage reactions are taking place simultaneously. If the transcription produces abortive RNAs similar in length to the desired product then it is *not possible* to separate these aborts from the cleaved product RNA utilizing the *cis*-cleavage procedure (see the gel of the transcription reaction shown in Fig. 5B). Since abortive initiation by T7 RNA polymerase generally does not produce RNA products >30 nt

long, longer RNAs produced by cleavage *in cis* will usually not be contaminated with these abortive transcripts. Thus for production of shorter RNAs (<40 nt), we recommend using *trans* cleavage with an initial purification of the transcription reaction to separate aborts from N and N+1 RNA products. However for longer RNAs, either the *cis* or *trans* cleavage should give comparable results in terms of purity of the desired product. For production of $^{13}C/^{15}N$-labeled RNA for NMR studies, we always employ cleavage *in trans* because the cleaving hammerhead ribozyme can be synthesized separately, thus avoiding incorporating the expensive labeled NTPs into the *cis* ribozyme that is not the desired product. The production of a second RNA is somewhat more effort but this is compensated for by the higher yields and simplified purification with the *trans* cleaving system. In addition, we routinely recycle the *trans*-cleaving hammerhead and have used the same ribozyme for large-scale cleavage reactions on three different RNA substrates (unpubl.).

A nonribozyme method for production of homogenous-length RNA transcripts involves sequence-specific cleavage of the transcript by RNase H (Lapham & Crothers, 1996). In this procedure a complementary oligonucleotide is synthesized as a chimera using 2′-*O*-methyl-nucleotides except for 4–5 deoxynucleotides at the 5′-terminus. This oligomer is annealed to the RNA transcript, making it a target for RNAse H cleavage of the RNA strand immediately across from the 5′-terminal deoxynucleotide. Chimeric DNA/2′-*O*-methyl-RNA oligonucleotides can be designed for either the 5′- or 3′-terminus with no sequence limitations, which makes this a potentially versatile method for generating homogenous-length RNA. However a major problem with this technique is that different sources of RNAse H yield different cleavage sites in the chimera (Lapham et al., 1997). In addition, directed cleavage of the DNA–RNA duplex requires stoichiometric levels of the chemically synthesized chimeric oligonucleotide, which can be relatively expensive for large-scale production of RNA.

Doudna and coworkers have also employed *trans* cleavage by the VS ribozyme to produce RNA with homogeneous 3′-termini (Ferré D'Amaré & Doudna, 1996; Doudna, 1997). In this procedure, the RNA is initially transcribed with the 24-nt VS ribozyme-binding sequence included as an extension to the 3′-terminus. The VS ribozyme is then added *in trans* (or cotranscribed) and the initial RNA transcript is cleaved to the desired length. Because the VS ribozyme cleaves at any base after C (5′-CN-3′), it allows processing of RNAs with 3′-terminal G residues that are not cleavable with hammerhead RNAs. Thus this VS ribozyme system is complimentary to the *trans*–hammerhead cleavage presented here. Doudna and coworkers employed denaturing PAGE for purification of RNAs generated by the VS ribozyme, but with proper design of the substrate, ribozyme, and products, it should be possible to purify RNAs generated by *trans* cleavage of the VS ribozyme by the anion-exchange HPLC methods described here. In conclusion, *trans* cleavage of RNA transcripts by hammerhead or VS ribozymes combined with purification of the RNA by anion-exchange HPLC allows production of milligram quantities of homogenous-length RNAs for biochemical or structural studies.

MATERIALS AND METHODS

Initial purification of substrate and hammerhead RNAs

Substrate and hammerhead RNA were prepared by in vitro transcription from synthetic DNA templates as previously described (Milligan et al., 1987; Batey et al., 1992; Nikonowicz et al., 1992). The DNA templates were purchased from PerSeptive Biosystems or Integrated DNA Technology and purified by denaturing 20% PAGE prior to use in transcription. After transcription, the RNA was subjected to an initial purification by either preparative denaturing 20% PAGE or anion-exchange HPLC (detailed below). At the initial purification stage, 2–5 mg RNA were separated with each gel or HPLC injection. The yield of RNA was quantitated by measuring the absorbance at 260 nm.

Analytical scale *trans* cleavage with hammerhead ribozyme

Cleavage conditions were optimized in 50-μL reactions containing 50 μM substrate RNA, 50 μM hammerhead RNA, 40 mM Tris, 100 mM NaCl, 2 mM EDTA, pH 7.4. Hammerhead and substrate RNAs were annealed by heating at 85 °C for 3 min and then cooled to room temperature over 30 min. Cleavage of the substrate was initiated by the addition of $MgCl_2$ to a final concentration of 25 mM, and reactions were incubated at 37 °C for 2–12 h. At selected time intervals, 5-μL aliquots were removed from the cleavage reaction and quenched by addition of 1 vol of denaturing gel loading dye (87% formamide containing 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). Samples were fully denatured by heating at 95 °C for 3 min and then separated by denaturing 20% PAGE. Analytical gels were developed with Stains-All (Kodak) and imaged on a light box equipped with a digital camera (Alpha Innotech, AlphaImager 2000).

Large-scale *trans* cleavage with hammerhead ribozyme

Conditions were scaled up linearly for the multi-milligram cleavage reaction. Typically, 10–25 mg substrate RNA were converted in a 30–50-mL reaction. Hammerhead and substrate RNA, buffer, NaCl, and EDTA were mixed and annealed as above. Cleavage was again initiated by the addition of $MgCl_2$ and reactions were incubated at 37 °C for 2–4 h. An aliquot from the large-scale reaction was removed after 2 h and quickly separated on a denaturing 20% analytical gel to ensure that the reaction had gone to completion. Large-scale

reactions were quenched by the addition of 50 mM EDTA or ethanol precipitation.

Large scale purification of RNA by denaturing PAGE

RNAs were purified on large-scale 20% denaturing PAGE as previously described (Batey et al., 1992; Nikonowicz et al., 1992). When these gels are used to separate medium-sized RNAs that differ by only 1 nt, only ~1 mg can be loaded per preparative gel. However gels loaded with 2–5 mg RNA still had adequate resolution for the applications here. This is because single-nucleotide resolution is not required in the initial purification or for purification of the hammerhead-cleaved product, where the different types of RNAs (product, substrate, and hammerhead) differ by >5 nt. Bands were visualized by UV shadowing, excised, and ground with a sterile glass rod. RNA was isolated from the gel pieces by soaking in a 0.3-M sodium acetate (pH 5.3), 2-mM EDTA solution followed by filtration through a 0.45 μM filter unit and then ethanol precipitation. The *trans*-cleaving hammerhead RNA was separated, isolated, and recycled for use in subsequent cleavage reactions.

Large scale purification by anion-exchange HPLC

All HPLC separations were performed on a Waters HPLC system equipped with an automated gradient controller, Model 486 detector, two Model 515 pumps, a Timberline column heater, and a Dionex NucleoPac PA100 (9 × 250 mm) HPLC column. Data were processed using the Dynamax software package (Rainin Instrument Co.). The column was heated to 85–90 °C, and buffers were (A) 25 mM Tris, 2 mM EDTA, pH 8.0 and (B) 25 mM Tris, 2 mM EDTA, 1 M NH_4Cl, pH 8.0. RNA eluted at ~400–800 mM NH_4Cl. Typical gradients for the initial purification after transcription (Gradient I, flow rate = 5 mL/min) and the final purification after hammerhead cleavage (Gradient II, flow rate = 7 mL/min) are given in Figures 4 and 5, respectively, although gradient conditions should be optimized to increase the resolution for each specific RNA system. Following HPLC purification, the RNA was concentrated and exchanged into appropriate buffers using either Centricon-3 concentrators or an Amicon ultrafiltration apparatus.

MALDI-TOF mass spectroscopy

RNA was desalted prior to mass spectral analysis by two ethanol precipitations from 0.4 M ammonium acetate solution followed by repeated lyophilization from distilled water to remove excess ammonium acetate. Either a 25-nt DNA (PerSeptive Biosystems, 7696 g/mol), or an 18-nt DNA (Integrated DNA Technology, 5468 g/mol) was used as an internal calibration standard. Wells on a gold-coated sample plate were spotted with 2.5 μL RNA (~100–200 μM), 2.5 μL internal DNA standard (~100–200 μM, 25 mM ammonium citrate, pH 8) and 5 μL matrix (50 mg/mL 3-hydroxypicolinic acid in 60% CH_3CN/40% 0.1 M ammonium formate, pH 9.4). The sample was removed from the sample plate and mixed with DOWEX cation exchange beads in the ammonium form to remove any remaining sodium or potassium ions. The sample was placed back in the well and allowed to air dry 30 min. Spectra were acquired with a PerSeptive Biosystems DE-STR Biospectrometry MALDI-TOF workstation in the linear-extraction, positive-ion mode. The accelerating voltage was set at 25,000 V and the delay was 300–450 ns.

NMR spectroscopy

RNA samples were dialyzed extensively against NMR buffers in Centricon-3 concentrators. ^1H and ^{31}P NMR spectra were acquired at 25 °C on a Varian INOVA 500 MHz spectrometer, equipped with either indirect or broadband probes.

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